

# Enhanced splice correction by 3', 5'-serinol and 2'-( $\omega$ -O-methylserinol) guarded OMe-RNA/DNA mixmers in cells

Venubabu Kotikam<sup>1</sup>, Andrey A Arzumanov<sup>2</sup>, Michael J Gait<sup>2,\*</sup>, and Vijayanti A Kumar<sup>1,\*</sup>

<sup>1</sup>Organic Chemistry Division; CSIR-National Chemical Laboratory; Pune, India; <sup>2</sup>Medical Research Council; Laboratory of Molecular Biology; Cambridge, UK

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**Abbreviations:** T<sup>R-AMP</sup>, 2'-(*R*)-aminomethoxypropyloxy-thymidine; AONs, antisense oligonucleotides

Development of artificial nucleic acids for therapeutic applications warrants that the oligomers be endowed with high specificity, enzymatic stability and with no/reduced off-target effects. The balance between strength of the duplex with target RNA and enzyme stability is therefore the key factor for the designed modification. The chiral serinol derivative combines the attributes of amino- and methoxy-substitution when at 2'-position and at 3'- and 5'-ends, effectively balancing the duplex stability and resistance to hydrolytic enzymes. The biological effect seen is the remarkable improvement in splice correction by the steric blocking antisense oligonucleotide with just 4 modified units, i.e. ~20% substitution with *R*-aminomethoxypropyloxy (*R*-AMP)-thymidine within the 2'-OMe 18mer sequence.

## Introduction

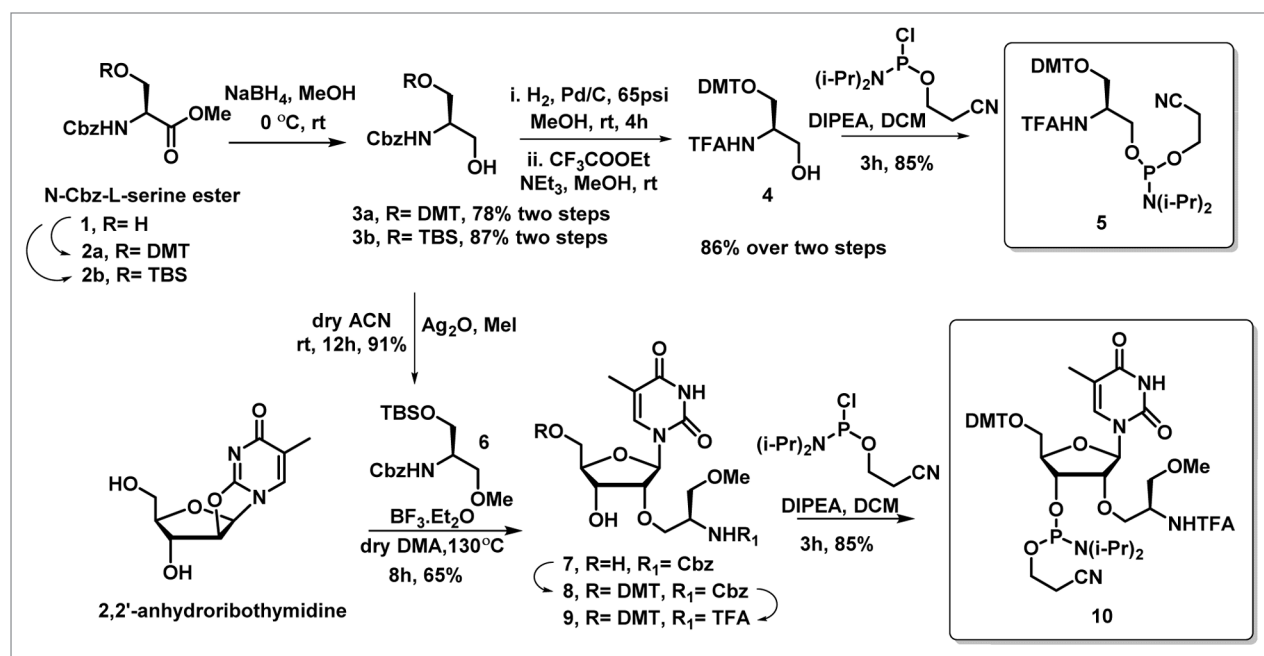
Zamecnik and Stephenson discovered the sequence specific biological activity of synthetic antisense oligonucleotides (AONs) with potential in therapeutics in 1978.<sup>1</sup> In their pioneering work, it was found that the efficacy of these AONs can be improved by capping the 3'/5'-ends which reduces the susceptibility of AONs toward enzymatic degradation. Tennant et al. had shown earlier in 1973, that nuclease resistant 2'-OMe-poly(A) exhibit higher inhibitory efficacy of over Poly(A) on murine oncornavirus production in tissue culture.<sup>2</sup> The chemistry of antisense AONs has progressed immensely over the last 4 decades when several analogs such as phosphorothioates (PS),<sup>3</sup> peptide nucleic acids (PNA),<sup>4</sup> morpholino nucleic acids (PMO)<sup>5</sup> were introduced as linkage modifiers and 2'-O-alkyl<sup>6</sup> derivatives such as 2'-OMe and 2'-O-methoxyethyl(MOE), 2'-F,<sup>7</sup> ANA,<sup>8</sup> LNA<sup>9</sup> etc. as sugar modifiers, for endowing nuclease resistance to oligomers as well as allowing increased efficiency of duplex formation (Fig. 1).<sup>3,10</sup> In spite of being diastereomeric mixtures at each phosphorus atom, the PS linkages have not yet found replacement because of their favorable pharmacological properties such as increasing half-life and improved binding to serum proteins in vivo, allowing greater availability of AONs to biological targets.<sup>11</sup> Several of these chemistries are being mixed in the recent years to gain maximum advantages in terms of reducing off-target effects, increasing specificity and potency of the AONs in various strategies such as RNase-H dependent antisense,<sup>3,10,12,13</sup> siRNA,<sup>14,15</sup> miRNA<sup>16,17</sup> or

splice switching antisense applications.<sup>18</sup> The recent literature also again points out the necessity to protect 3'-5' ends by enzymatically stable capping of AONs.<sup>14,16</sup>

It appears that among the plethora of modified AONs currently under evaluation, the promising AONs have some undesirable drawbacks, e.g., phosphorothioate AONs or OMe/LNA mixmers show non-sequence-specific effects due to nonspecific binding to untargeted proteins<sup>19</sup> or due to mismatched non-target recognition as a result of very high duplex stability of AON duplexes with target RNA.<sup>20</sup> The enzyme resistant phosphorothioate AONs are a mixture of diastereomers at every linker phosphorus atom and the separation of diastereomers is not easy.<sup>21</sup> Such AONs also show reduced binding efficiency to RNA. The enzyme resistant LNA analogs<sup>22,23</sup> such as c-OMe or c-Et also require several synthetic steps and separation of diastereomers during their synthesis. These shortcomings are indicative of the pressing need for efficient AON analogs that employ relatively simple chemistry, are chirally homogeneous but are still endowed with less toxic off-target effects and have higher efficiencies.

Recently, we designed an  $\omega$ -O-methylserinol derived 2'-O-(*R*-2-amino-3-methoxypropyl) (2'-*R*-AMP) modification of uridine which combines the characteristics of 2'-MOE and 2'-aminopropyl substitution in a stereospecific manner.<sup>24</sup> The amino pendant group in the minor groove as in 2'-O-(2-aminoethyl)- substituent was earlier found to be responsible for displacing the essential cations in the hydrolytic enzyme binding site, thus inhibiting the enzyme activity.<sup>25</sup> As expected, when

\*Correspondence to: Michael J Gait; Email: mgait@mrc-lmb.cam.ac.uk; Vijayanti A Kumar; Email: va.kumar@ncl.res.in  
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**Scheme 1.** Synthesis of universal end cap and *R*-AMP thymidine monomers.

(2'-*R*-AMP) modification was introduced in DNA oligomers, the AONs were found to be as good as 2'-MOE oligomers in terms of efficiency of duplex formation, along with much higher resistance to enzymatic degradation compared with 2'-MOE oligomers.<sup>24</sup>

In this article, we now present the synthesis of protected-(2-amino-1,3-dihydroxypropyl) monomer unit from L-serine, as a universal serinol cap to the oligomers at 3', 5'-ends and the 2'-*O*-*R*-AMP-ribothymidine monomer to increase the enzymatic resistance of 2'-OMe RNA without disturbing the efficacy of duplex formation. Thymidine is known to show slightly better duplex stability compared with uridine derivatives.<sup>26</sup> We further show here that the 3'- and 5'-capped 2'-OMe-AON with ~20% evenly dispersed modified  $T^{R-AMP}$  units is as effective as a LNA-OMe mixer containing ~40% LNA for antisense applications in steric blocking splice correction of an aberrant  $\beta$ -globin gene, using the luciferase reporter system developed by Kole and colleagues.<sup>27</sup>

## Results and Discussion

The synthesis of the universal end-capping monomer **5** and the 2'-*O*-*R*-AMP-thymidine monomer **10** is outlined in Scheme 1. The primary hydroxyl group of serine derivative **1** was protected as TBDMS (**2a**) or DMT (**2b**) ethers, followed by ester reduction to produce unsymmetrized diols **3a** and **3b**, carrying respective protecting groups. Compound **3a** was then hydrogenated to get a free amine, which was again protected as the TFA derivative (**4**), and converted into the protected serinol based amidite **5**, compatible with solid phase phosphoramidite oligomer synthesis. Alcohol **3b** was methylated (**6**) and was used to functionalize the ribothymidine to get the thymidine derivative **7** via opening of

2,2'-anhydro ring of 2,2'-anhydroribothymidine, as reported earlier for the 2,2'-anhydrouridine.<sup>24</sup> The primary hydroxyl group in **7** was protected with DMT (**8**), the primary amino group protection was changed to TFA (**9**), and was finally converted to amidite **10** compatible with solid phase phosphoramidite oligomer synthesis. All the new compounds were adequately characterized using NMR and HRMS analysis. The sequences synthesized using the amidites **5** and **10** were used for correction of splicing in thalassemic pre-mRNA by antisense oligonucleotides.<sup>28</sup> The sequence DNA1 is a control sequence without any end capping. Sequences DNA1Sa and DNA1Sb correspond to the single and double end capping (by employing monomer **5**) at both 3'- and 5'-ends. OMe-RNA is the 2'-OMe derivatised RNA sequence. OMe-RNAsa and OMe-RNASb are the single and double serinol capped OMe-RNA sequences, respectively. In the sequence OMe-RNA4-dTSb, four 2'-OMe-uridine residues are replaced by 2'-*O*-aminomethoxypropyl-ribothymidine derivative **10** ( $T^{R-AMP}$ ) at defined positions. The sequences thus synthesized are listed in Table 1. The single and double capping of the sequences with the serinol derivative was performed to assess the resistance offered by the capping to phosphodiesterase enzyme. All the sequences were purified by reverse phase HPLC and were characterized by MALDI-ToF mass analysis (Table 1).

We further explored in vitro studies regarding the protection of these newly synthesized oligomers against hydrolytic cleavage and compared the results with unmodified DNA1 and OMe-RNA. We digested these sequences with SVPD under conditions reported earlier.<sup>24,29</sup> The products of the digestion were analyzed by RP-HPLC and percent intact AON was plotted against time to understand the degradation pattern for all the oligomers (Fig. 2). The terminal serinol units were able to completely guard the 2'-OMe-RNA oligomers (OMe-RNAsa and OMe-RNASb)

**Table 1.** The sequences synthesized, their MALDI-ToF mass analysis and UV  $T_m$  values with complementary RNA and RNA with single mismatch

No	Sequence and Sequence code	MALDI-ToF mass Cald/Obsd	UV- $T_m$ °C cRNA/mmRNA
1	CCTCTTACCT CAGTTACA (DNA1)		56.6/50.4
2	<b>S</b> CCTCTTACCT CAGTTACA <b>S</b> (DNA1Sa)	5672.9/5690.3	58.3/50.4
3	<b>SS</b> CCTCTTACCT CAGTTACA <b>SS</b> (DNA1Sb)	5979.0/6002.0	58.4/48.7
4	CCUCUUACCU CAGUUACA (OMe-RNA)	5823.0/5824.4	77.0/66.2
5	<b>S</b> CCUCUUACCU CAGUUACA <b>S</b> (OMe-RNASa)	6129.0/6151.4	78.2/67.9
6	<b>SS</b> CCUCUUACCU CAGUUACA <b>SS</b> (OMe-RNASb)	6435.1/6453.0	76.8/65.4
7	<b>SS</b> CCT <sup>AMP</sup> CU <sup>AMP</sup> ACCT <sup>AMP</sup> CAGU <sup>AMP</sup> ACA <b>SS</b> (OMe-RNA4-dTSb)	6786.6/6787.8	76.4/64.0

cRNA2 UGUAACUGAG GUAAGAGG; mmRNA3 UGUAACUGCG GUAAGAGG; A/C/G/U RNA or 2'-OMeRNA nucleotides; **S** is a serinol capping unit; **T<sup>AMP</sup>** is a 2'-O-(2-amino-3-methoxypropyl)ribothymidine unit.

against hydrolytic cleavage by SVPD for up to 24 h compared with the uncapped DNA or OMe-RNA sequences. After digestion with SVPD for 24h, both the DNA capped sequences DNA1Sa and DNA1Sb were degraded at least 70 and 50%, respectively. Having confirmed the enzymatic stability conferred to these sequences by end capping and 2'-OMe introduction, we studied the effect of these modifications on UV- $T_m$  studies with complementary cRNA2 and single base mismatched mmRNA3. It was observed that the end capping as well as the incorporation of **T<sup>AMP</sup>** units have negligible effect on the binding efficiency of the 2'-OMe oligomer. The mismatch discrimination was found to be slightly better or equal to the 2'-OMe oligomer (Table 1), indicating that the amino pendant groups do not have any additional non-specific electrostatic interactions with target RNA. The stabilization effect of **T<sup>AMP</sup>** introduction is comparable to OMe substitution.

For biological experiments, to have maximum effects, we chose the sequences OMe-RNASb and OMe-RNA4-dTSb, each having two serinol caps at both 3'- and 5'-ends, which were competent for duplex formation as well as being enzyme-resistant. Cells expressing luciferase pre-mRNA, interrupted by an aberrantly spliced  $\beta$ -globin intron, HeLa pLuc705, were used to monitor the splice-switching activity of the modified oligonucleotides. By using these cells, various AONs have been evaluated by measuring induced luciferase activity as a tool to verify splice switching efficiency and specificity.<sup>28</sup> OMe-RNASb and OMe-RNA4-dTSb were used in this study in comparison with OMe-RNA, and also with an LNA-OMe mixmer, previously used in recent steric blocking applications<sup>17,30</sup> and the detection was of the expression of functional luciferase in the form of RLU/ $\mu$ g protein expression.

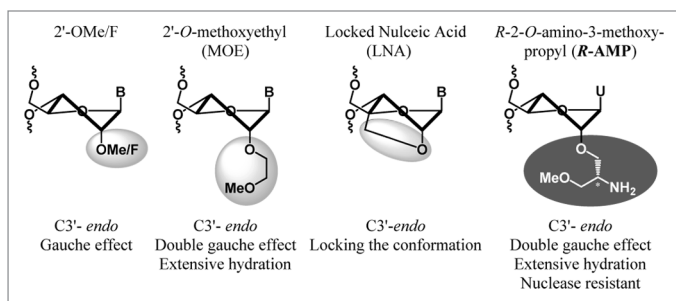
The results shown in Figure 3 clearly indicate that the dose-dependent luciferase expression in the cells treated with serinol-end-capped OMe-RNASb is better than that for the uncapped OMe-RNA. The expression was found to be even better for the sequence OMe-RNA4-dTSb, which was modified further by incorporation of four **T<sup>AMP</sup>** units (i.e. ~20% substitution of U<sup>OMe</sup> with 2'-O-**T<sup>AMP</sup>**). The expression of corrected fluorescent protein was found to be as good as or even slightly better than an LNA/OMe mixmer containing about ~40% LNA substitution. LNA substitution is known to impart very high stability to the duplexes with cRNA (~5 °C/LNA unit).<sup>9,31</sup> In addition, LNA/

OMe mixmers have been previously reported to show high nuclear steric blocking activity following transfection whereas OMe-RNA was inactive.<sup>30</sup> The present modification, in the sequence OMe-RNA4-dTSb, does not have the advantage/disadvantage of LNA in the context of additive duplex stability per modified unit but nevertheless is still able to show an ability to enter the cell nucleus and give high splice correction activity. As is evident from Figure 3, the end capping of OMe-RNA is not enough to obtain good activity but the interspersed four **T<sup>AMP</sup>** units enhance the luciferase expression significantly and to a level obtained by as much as seven LNA units, which is remarkable. This could be due to the balanced duplex stability and protection against enzymatic susceptibility endowed by internal **T<sup>AMP</sup>** units in addition to the end capping. We have also shown by UV- $T_m$  studies, that the single mismatch discrimination is as high as that shown by OMe-RNA oligomer while forming a duplex with the target RNA and therefore off-target effects might also be less compared with the LNA-OMe mixmer sequences, where sometimes mismatched bases in the target RNA can be tolerated.<sup>20</sup> The oligomer OMe-RNA4-dTSb with 8 additional amino groups, which partially neutralizes the negative charge, was used also in luciferase activity experiments without transfection agent, but no activity was observed (Please see **Supplemental Materials**). This is perhaps not surprising since there is still a net negative charge in the oligomer.

Currently used therapeutic oligomers employ phosphorothioate substitution for avoiding the susceptibility of the oligomers to hydrolytic enzymes and also to enhance cellular and in vivo activity, and it would be interesting to study if our novel strategy of 2'- substitution presented here can be used to improve cell uptake further and possibly even to allow one to avoid (or at least reduce) the requirement for phosphorothioate substitution. The results presented in this article may have implications in other diseases including cystic fibrosis, muscular dystrophies, cancers, and several neurological disorders where there are associated mutations affecting the splicing process.<sup>3</sup>

## Materials and Methods

All the non-aqueous reactions were performed under the inert atmosphere of Nitrogen/ Argon and the chemicals used were of laboratory or analytical grade. All solvents used were

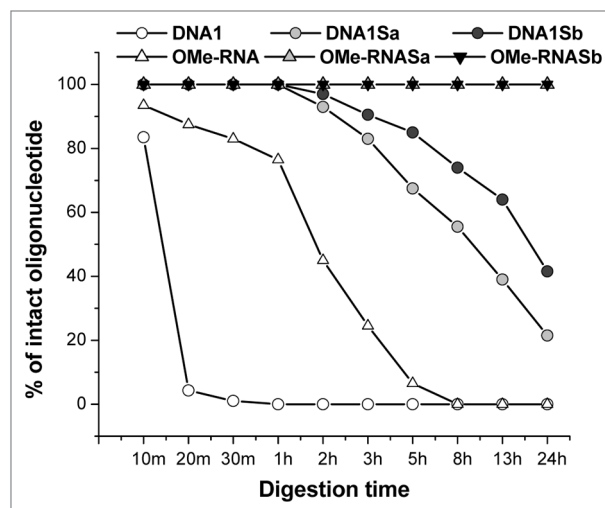


**Figure 1.** Examples of 2'-Sugar modified AONs for potential applications.

dried and distilled according to standard protocols. TLCs were performed on pre-coated silica gel GF254 sheets (Merck 5554). All reactions were monitored by TLC and usual work-up implies sequential washing of the organic extract with water and brine followed by drying over anhydrous sodium sulfate and evaporation of the organic layer under vacuum. Column chromatographic separations were performed using silica gel 100–200 mesh (Merck) or 230–400 mesh (Merck) and using the solvent systems EtOAc/Petroleum ether and MeOH/DCM.  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectra were obtained using Bruker AC-200, AC-400 or AC-500 NMR spectrometers. The chemical shifts are reported in delta ( $\delta$ ) values and referred to internal standard TMS for  $^1\text{H}$ . HRMS-mass spectra were recorded on a Finnigan-Matt mass spectrometer. cDNA sequences were synthesized on Bioautomation Mer-Made 4 DNA synthesizer using standard  $\beta$ -cyanoethyl phosphoramidite chemistry. DNA sequences were also analyzed and purified under the same conditions with the increasing gradient of acetonitrile in 0.1N triethylammonium acetate of pH 7.0. The MALDI-TOF spectra were recorded on AB Sciex TOF/TOF<sup>TM</sup> series explorer<sup>TM</sup> 72085 instrument; THAP (2, 4, 6-trihydroxyacetophenone) was used as the matrix for DNA samples characterization. Thermal denaturation experiments were performed on Varian Cary-300 UV-Vis spectrophotometer fitted with a peltier-controlled temperature programmer and a water circulator, at the temperature ramping 0.5  $^\circ\text{C}/\text{min}$  and the absorbance is recorded at 260 nm for every 0.5  $^\circ\text{C}$  rise in temperature. OMe-RNA/LNA and OMe-RNA/LNA/scr oligomers were obtained from RiboTask ApS, Odense, Denmark.

#### Methyl *N*-[(benzyloxy)carbonyl]-*O*-dimethoxytrityl-L-serinate **2a**

*N*-Cbz-L-serine methyl ester **1** (19.7 mmol, 5.0 g) was dissolved in dry pyridine (25 mL) and DMT-Cl (21.6 mmol, 7.3 g) and catalytic amount of DMAP (~30 mg) were added. Reaction mixture was kept for stirring at room temperature for 4 h. Pyridine was removed under reduced pressure and the residue was diluted with EtOAc. 10% aqueous  $\text{NaHCO}_3$ , water and brine solution wash were given to the organic layer. The organic layer was dried over anhydrous  $\text{Na}_2\text{SO}_4$  and concentrated to dryness. Crude compound was column purified (eluted in 20% EtOAc in petroleum ether) to get **2a** as a pale thick liquid in 91% yield.  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 200 MHz):  $\delta$  3.34–3.39 (dd, 1H,  $J$  = 2.65 and 9.09 Hz), 3.54–3.60 (dd, 1H,  $J$  = 2.78 and 9.09 Hz), 3.76 (s,



**Figure 2.** Percent intact AON plotted against time.

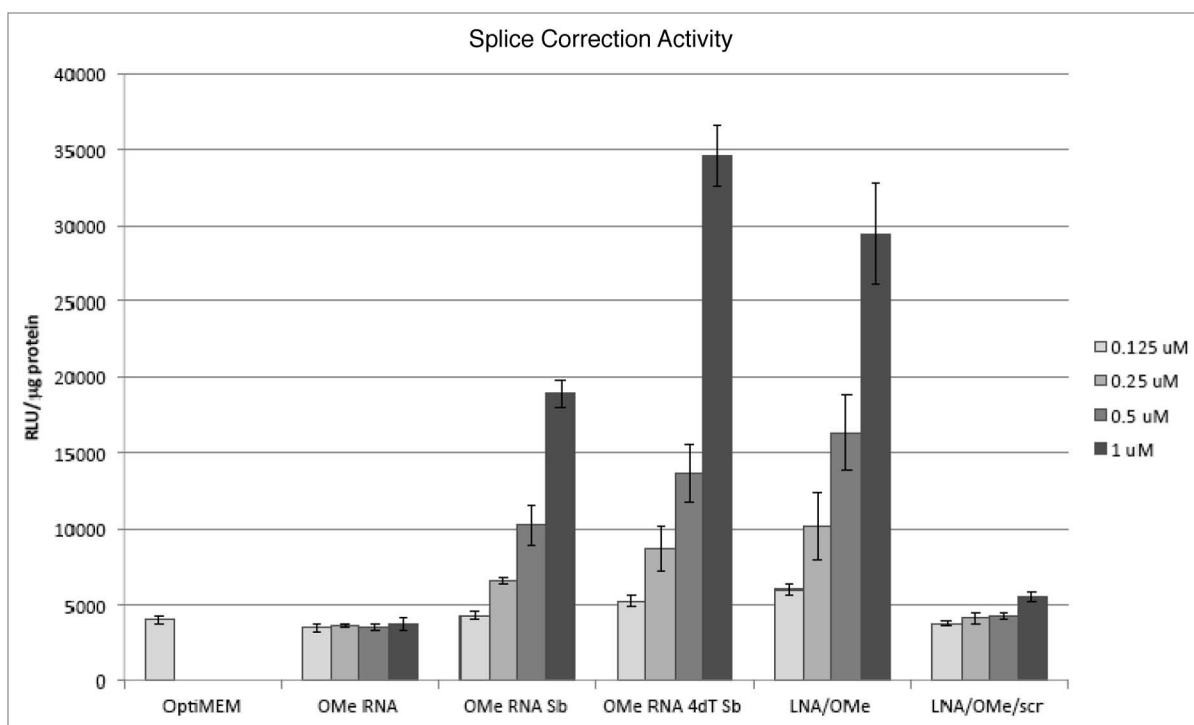
9H), 5.11 (s, 2H), 5.67 (d, 1H,  $J$  = 8.59 Hz), 6.78–6.82 (m, 4H), 7.22 (m, 5H), 7.34 (m, 9H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 50 MHz):  $\delta$  52.0, 54.2, 54.8, 63.3, 66.6, 77.2, 85.7, 112.9, 126.6, 127.6, 127.68, 128.2, 128.9, 129.6, 135.1, 35.2, 136.0, 144.1, 155.6, 158.3, 170.8 HRMS (EI) Mass calculated for  $\text{C}_{33}\text{H}_{33}\text{O}_7\text{NNa}$  ( $M+\text{Na}$ ) 578.2149, found 578.2148.

#### Benzyl (*R*)-(1-[(dimethoxytrityl)oxy]-3-hydroxypropan-2-yl)carbamate **3a**

Compound **2a** (18.0 mmol, 10 g) was dissolved in MeOH (500 mL) and  $\text{NaBH}_4$  (72 mmol, 2.7 g) was added fraction-wise at 0  $^\circ\text{C}$  for a period of 40 min and the mixture was kept for stirring at room temperature for another 6–8 h. Excess  $\text{NaBH}_4$  was quenched with saturated  $\text{NH}_4\text{Cl}$  solution, followed by the removal of MeOH under reduced pressure. The crude reaction mixture was extracted with EtOAc. The organic extract was washed with brine solution and dried over anhydrous  $\text{Na}_2\text{SO}_4$ . EtOAc was removed under reduced pressure to give the crude product and was purified through column chromatography (eluted in 25–30% EtOAc in petroleum ether) to yield **3a** as a colorless liquid in 85% (7.8 g).  $^1\text{H}$  NMR ( $\text{CDCl}_3$ , 200 MHz):  $\delta$  2.39 (bs, 1H), 3.29 (d, 2H,  $J$  = 3.67 Hz), 3.73–3.79 (m, 8H), 5.10 (s, 2H), 5.32 (d, 1H,  $J$  = 7.58 Hz), 6.79–6.84 (m, 4H), 7.15–7.40 (m, 14H).  $^{13}\text{C}$  NMR ( $\text{CDCl}_3$ , 50 MHz):  $\delta$  52.5, 54.9, 62.9, 66.6, 86.2, 113.0, 126.7, 127.7, 127.8, 128.3, 129.7, 130.5, 136.2, 144.4, 156.3, 158.3. HRMS (EI) Mass calculated for  $\text{C}_{32}\text{H}_{33}\text{O}_6\text{NNa}$  ( $M+\text{Na}$ ) 550.2200, found 550.2198.

#### (*R*)-*N*-(1-[(dimethoxytrityl)oxy]-3-hydroxypropan-2-yl)-2,2,2-trifluoroacetamide **4**

Compound **3a** (8.7 mmol, 4.6 g) was dissolved in MeOH (20 mL) followed by the addition of 10% Pd-C (10% w/w, 0.46 g). The reaction mixture was subjected to catalytic hydrogenation at 60 psi of hydrogen pressure for 5 h. After the TLC analysis, reaction mixture was filtered over celite and the removal of methanol in vacuo gave free amine. Without further purification amine was subjected to trifluoroacetyl protection. To the crude amine (8.4 mmol, 3.3 g) dissolved in MeOH (30 mL),  $\text{NEt}_3$  (12.7 mmol, 1.7 mL) was added. Ethyltrifluoroacetate (10.1



**Figure 3.** Concentration dependent splice correction activity of oligonucleotides (Table 1) in this study. Control  $cC^L uC^L aC^L caT^L ugA^L cA^L a$  (LNA/OMe),  $cT^L cuC^L uC^L aC^L caT^L ugA^L cA^L a$  (LNA/OMe/scr).

mmol, 1.2 mL) was added to reaction mixture and the mixture was kept for stirring at room temperature for 8h. MeOH was removed on rota evaporator and the reaction mixture was diluted with EtOAc. The organic layer was washed with water and 5% aq.  $NaHCO_3$  and the organic layer was dried over anhydrous  $Na_2SO_4$ , concentrated in vacuo. Crude compound was subjected to column purification to furnish **4** (eluted in 17% EtOAc in petroleum ether) in 86% yield over two steps.  $^1H$  NMR ( $CDCl_3$ , 200 MHz):  $\delta$  2.39 (bs, 1H), 3.37(d, 2H,  $J = 4.42$  Hz), 3.68–3.75 (m, 1H), 3.78 (s, 6H), 3.83–3.90 (m, 1H), 4.05–4.14 (p, 1H), 6.82–6.86 (m, 4H), 7.26–7.40 (m, 9H).  $^{13}C$  NMR ( $CDCl_3$ , 50 MHz):  $\delta$  51.4, 55.0, 61.9, 62.1, 77.2, 86.5, 113.2, 126.9, 127.7, 127.9, 129.7, 135.2, 144.1, 158.5. HRMS (EI) Mass calculated for  $C_{26}H_{26}O_5NF_3Na$  (M+Na) 512.1655, found 512.1653.

#### Serinol derived phosphoramidite **5**

To the compound **4** (2.0 mmol, 1 g) dissolved in dry DCM (12 mL), DIPEA (3.6 mmol, 0.6 mL) was added. 2-cyanoethyl- $N,N$ -diisopropyl-chlorophosphine (2.4 mmol, 0.5 mL) was added to the reaction mixture at 0 °C and continued stirring at room temperature for 1.5 h. The contents were diluted with DCM and washed with 5%  $NaHCO_3$  solution. The organic phase was dried over anhydrous  $Na_2SO_4$  and concentrated. The residue was re-dissolved in DCM and the compound was precipitated with n-hexane to obtain corresponding phosphoramidite **5** in 85% yield.  $^{31}P$  NMR (Acetonitrile,  $D_2O$  as external standard, 160 MHz):  $\delta$  149.17, 149.20. HRMS (EI) Mass calculated for  $C_{35}H_{43}O_6N_3F_3PNa$  (M+Na) 712.2734, found 712.2728.

**Benzyl (R)-(1-[(tert-butyldimethylsilyl)oxy]-3-hydroxypropan-2-yl)carbamate **3b****

$N$ -Cbz protected-L-serine-methyl ester **1** (39.5 mmol, 10 g) was dissolved in dry DCM (200 mL), followed by the addition of imidazole (98.8 mmol, 6.7 g) and TBS-Cl (47.4 mmol, 7.1 g). The reaction mixture was diluted with DCM and water wash, brine wash were given. Organic layer was dried over anhydrous  $Na_2SO_4$  and solvent was removed under reduced pressure to result the crude TBS protected ester, which was subjected  $NaBH_4$  reduction. The residue was dissolved in methanol (500 mL) and cooled to 0 °C, was added  $NaBH_4$  (150 mmol, 5.6 g) fraction wise for a period of 1 h and then continued stirring at room temperature for another 6 h. Excess  $NaBH_4$  was quenched with saturated  $NH_4Cl$  solution, followed by the removal of MeOH under reduced pressure and extracted with EtOAc. The organic extract was washed with brine and dried over anhydrous  $Na_2SO_4$ . EtOAc was removed under reduced pressure to give the crude product and was purified through column chromatography (eluted in 15% EtOAc in petroleum ether) to yield **3b** in 87% (11.6 g) over two steps.  $^1H$  NMR ( $CDCl_3$ , 200 MHz):  $\delta$  0.05 (s, 6H), 0.88 (s, 9H), 2.64 (bs, 1H), 3.66–3.84 (m, 5H), 5.11 (s, 2H), 5.38–5.41 (m, 1H), 7.35–7.38 (m, 5H).  $^{13}C$  NMR ( $CDCl_3$ , 50 MHz):  $\delta$  -5.6, 18.1, 25.7, 53.0, 63.0, 63.4, 66.7, 128.0, 128.4, 136.2, 158.2. HRMS (EI) Mass calculated for  $C_{17}H_{30}O_4NSi$  (M+H) 340.1939, found 340.1945.

**Benzyl (R)-(1-[(tert-butyldimethylsilyl)oxy]-3-methoxypropan-2-yl)carbamate **6****

To a stirred solution of **3b** (29.4 mmol, 10 g) and MeI (147.4 mmol, 9.5 mL) was added  $Ag_2O$  (73.5 mmol, 16.9 g). The reaction mixture was stirred at room temperature for 12 h, filtered and filtrate was concentrated under reduced pressure.

Crude compound was purified through column chromatography (eluted in 5% EtOAc in petroleum ether) to result **6** in 91% (9.4 g) yield. <sup>1</sup>H NMR (CDCl<sub>3</sub>, 200 MHz): δ 0.05 (s, 6H), 0.88 (s, 9H), 3.33 (s, 3H), 3.37–3.77 (m, 4H), 3.78–3.86 (m, 1H), 5.10 (s, 2H), 7.35–7.38 (m, 5H). <sup>13</sup>C NMR (CDCl<sub>3</sub>, 100 MHz): δ -5.6, 18.1, 25.7, 51.5, 58.8, 61.3, 66.6, 70.4, 128.0, 128.4, 136.4, 155.9. HRMS (EI) Mass calculated for C<sub>18</sub>H<sub>32</sub>O<sub>4</sub>NSi (M+H) 354.2095, found 354.2089.

#### 2'-functionalization of 2,2'-anhydroribothymidine 7

Desiccated 2,2'-anhydroribothymidine (6.2 mmol, 1.5 g) was dissolved in dry DMA (10 mL) followed by the addition of BF<sub>3</sub>·OEt<sub>2</sub> (12.5 mmol, 1.5 mL) under argon atmosphere. After 2 min the -TBS protected silyl ether **5** (18.7 mmol, 6.6 g) was added and stirred at 130 °C for 8 h. DMA was removed partially on rota evaporator followed by the dilution with MeOH, the reaction mixture was then column purified (eluted in 75% EtOAc in petroleum ether) to yield 65% of **7** as white solid. <sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>) δ<sub>H</sub> 1.83 (s, 3H), 2.06 (s, DMA, COCH<sub>3</sub>), 2.91 (s, 3H), 2.99 (s, 3H), 3.31 (s, 3H), 3.47 (m, 2H), 3.65–4.12 (m, 10H), 4.29 (m, 1H), 5.06 (s, 2H), 5.77 (d, J = 3.22 Hz, 1H), 7.30 (m, 5H), 7.64 (s, 1H), 9.97 (bs, 1H); <sup>13</sup>C NMR (50 MHz, CDCl<sub>3</sub>) δ<sub>C</sub> 12.1, 21.2 (DMA, -CH<sub>3</sub>), 29.4, 35.0, 37.8 (DMA, -N-CH<sub>3</sub>), 49.9, 58.7, 60.4, 66.5, 68.3, 69.7, 71.1, 77.2, 82.1, 84.4, 88.1, 110.4, 127.7, 127.8, 128.2, 136.1, 136.8, 150.5, 156.3, 164.3, 171.0 (DMA, -CO); HRMS (EI) Mass calculated for C<sub>22</sub>H<sub>29</sub>N<sub>3</sub>O<sub>9</sub>Na (M+Na) 502.1796, found 502.1800.

#### 5'-O-Dimethoxytrityl-2'-O-[N-benzyloxycarbonyl-2'-O-R-(2-amino-3-methoxypropyl)] ribothymidine 8

2'-O-functionalized ribothymidine **7** (3.0 mmol, 1.5 g) dissolved in dry pyridine (10 mL) and was added DMT-Cl (3.3 mmol, 1.1 g) and catalytic amount of DMAP (~20 mg). Reaction mixture kept for stirring at room temperature for 5–6 h. Pyridine was removed under reduced pressure and the residue dissolved in EtOAc. 10% aqueous NaHCO<sub>3</sub>, water and brine wash were given to the organic layer. The organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated to dryness. Crude compound was column purified (eluted in 60% EtOAc in petroleum ether) to result **8** in 81% yield as a white foam. <sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>) δ<sub>H</sub> 1.36 (s, 3H), 3.34 (s, 3H), 3.37–3.58 (m, 5H), 3.78 (s, 6H), 3.97–4.09 (4H), 4.41 (m, 2H), 5.09 (s, 2H), 6.81 (m, 2H), 7.28–7.65 (m, 12H), 7.65 (s, 1H), 9.51 (bs, 1H); <sup>13</sup>C NMR (50 MHz, CDCl<sub>3</sub>) δ<sub>C</sub> 11.7, 29.6, 50.1, 55.1, 59.0, 62.1, 66.8, 68.9, 70.7, 71.5, 77.2, 82.9, 83.5, 86.8, 87.1, 111.0, 113.2, 127.0, 127.9, 128.1, 128.4, 130.0, 135.2, 136.2, 144.2, 150.4, 156.2, 158.6, 164.0; HRMS (EI) Mass calculated for C<sub>43</sub>H<sub>47</sub>N<sub>3</sub>O<sub>11</sub>Na (M+Na) 804.3103, found 804.3105.

#### 5'-O-Dimethoxytrityl-2'-O-(N-trifluoroacetyl-2'-O-R-(2-amino-3-methoxypropyl)) ribothymidine 9

The 5'-DMT protected 2'-O-functionalized thymidine derivative **8** (2.6 mmol, 2.1 g) was dissolved in MeOH (15 mL) and was added 10% Pd-C (10% w/w, 0.21 g). Then reaction mixture was subjected to catalytic hydrogenation at 65 psi of

hydrogen pressure for 5 h. After the TLC analysis Pd-C was removed by filtration over celite and concentration of the filtrate in vacuo gave free amine. Without further purification amine was subjected to trifluoroacetyl protection. Crude amine (2.5 mmol, 1.6 g) was dissolved in MeOH (15 mL), followed by the addition of NEt<sub>3</sub> (3.7 mmol, 0.54 mL). Ethyltrifluoroacetate was added and the reaction mixture kept for stirring at room temperature for 10 h. MeOH was removed on rota evaporator and the reaction mixture was diluted with EtOAc. To the EtOAc containing reaction mixture gave water wash and 5% aq. NaHCO<sub>3</sub> wash. Organic layer was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> concentrated in vacuo. Crude compound was column purified (eluted in 60% EtOAc in petroleum ether) to yield **9** in 78% over two steps as a white foam. <sup>1</sup>H NMR (200 MHz, CDCl<sub>3</sub>) δ<sub>H</sub> 1.36 (s, 3H), 3.38 (s, 3H), 3.45–3.54 (m, 2H), 3.59 (d, J = 4.55 Hz), 3.79 (s, 6H), 3.84–3.89 (m, 1H), 4.00–4.11 (m, 3H), 4.31–4.49 (m, 2H), 5.95 (d, J = 2.91 Hz), 6.81 (m, 4H), 7.27–7.51 (m, 9H), 7.69 (s, 1H), 9.44 (bs, 1H); <sup>13</sup>C NMR (50 MHz, CDCl<sub>3</sub>) δ<sub>C</sub> 11.7, 49.3, 55.2, 59.1, 61.9, 68.8, 69.5, 70.5, 83.1, 83.5, 86.8, 87.3, 111.4, 113.1, 113.2, 127.1, 127.7, 127.7, 128.0, 129.0, 130.0, 135.0, 135.2, 144.2, 150.9, 158.6, 163.8; HRMS (EI) Mass calculated for C<sub>37</sub>H<sub>40</sub>F<sub>3</sub>N<sub>3</sub>O<sub>10</sub>Na (M+Na) 766.2558, found 766.2561.

#### Phosphoramidite 10

To a solution of **9** (1.3 mmol, 1 g) in dry DCM (15 mL) was added DIPEA (5.8 mmol, 1.0 mL). 2-cyanoethyl-N,N-diisopropyl-chloro phosphine (1.6 mmol, 0.35 mL) was added to the solution at 0 °C and reaction mixture was stirred at room temperature for 3 h. The contents were then diluted with DCM and washed with 5% NaHCO<sub>3</sub> solution. The organic phase was dried over anhydrous Na<sub>2</sub>SO<sub>4</sub> and concentrated to foam. The residue was redissolved in DCM and precipitated with n-hexane to yield corresponding phosphoramidite **10** in 82% as white foam. <sup>31</sup>P NMR (Acetonitrile, D<sub>2</sub>O as external standard, 400 MHz): δ 149.22, 149.68. HRMS (EI) Mass calculated for C<sub>46</sub>H<sub>58</sub>F<sub>3</sub>N<sub>3</sub>O<sub>11</sub>P (M+H) 944.3817, found 944.3824.

#### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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#### Supplemental Materials

Supplemental materials may be found here:  
[www.landesbioscience.com/journals/artificialdna/article/27279](http://www.landesbioscience.com/journals/artificialdna/article/27279)

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